DEFINITION OF A SPECIFIC INTERACTION BETWEEN THE EARLY T LYMPHOCYTE ACTIVATION 1 (Eta-1) PROTEIN AND MURINE MACROPHAGES IN VITRO AND ITS EFFECT UPON MACROPHAGES IN VIVO

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Although lymphocytes can be specifically activated by cells that express peptide/MHC complexes associated with infection by viruses or bacteria (1), they are not usually equipped to rapidly eliminate infected target cells. An effective immune response depends on the elaboration of factors ("lymphokines") that can attract macrophages and other effector cells to the site of infection and enhance their capacity to destroy ingested microbes (2, 3). An extensive effort to define such lymphokines has resulted in the description of a large number of factors in supernatant fluids of activated lymphocytes designated by acronyms associated with their activity; MMF (macrophage migration factor), MIF (migration inhibitory factor), MAF (macrophage activation factor), and MCF (macrophage cytotoxicity factor) (4–6).

To define genes that code for proteins secreted by activated T lymphocytes, we derived a subset of cloned cDNA inserts representing genes transcribed shortly after T cell activation. One such gene, designated *Eta-1* (Early T lymphocyte activation 1),[1] encodes a secreted protein which is associated with genetic resistance to in vivo infection by an obligate intracellular bacteria (7). Studies of the in vivo expression of *Eta-1* suggested that it might enhance bacterial resistance by affecting the ability of macrophages to migrate to sites of infection and/or to express bacteriocidal activity (7). We have studied the interaction of the eta-1 protein with macrophages in vitro and in vivo. We find that purified eta-1 binds specifically to murine macrophages through an Arg-Gly-Asp (RGD) containing domain and that in vivo administration of eta-1 results in a cellular infiltrate that is composed primarily of this cell type.

Materials and Methods

Cells. The mouse myelomonocytic cell line Wehi 3 and fibroblast cell line NIH3T3 were maintained in DME supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml

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[1] *Abbreviations used in this paper:* eta-1, early T lymphocyte activation 1; RPM, resident peritoneal macrophages.

streptomycin, and 10% heat-inactivated FCS. The generation and culture conditions of the $CD4^+$ T cell clones used in this report, 03, Ar5 and Ar5v, are described elsewhere (8, 9). To obtain a cell population enriched for macrophages, peritoneal fluid of BALB/c mice following intraperitoneal injection of 5 ml PBS was aspirated and 5×10^5 cells/ml was incubated at 37°C in 96-well tissue culture plates (Flow Laboratories, McLean, VA). 2 h later nonadherent cells were removed after wells were washed 5–6 times with PBS. Approximately 30–40% of the cells remained adherent and consisted of 95–99% macrophages according to Giemsa and peroxidase staining and are designated resident peritoneal macrophages (RPM). No $Thy\text{-}1^+$ cells were detectable by immunofluorescence and 90–95% of the cells were murine $Mac\text{-}3^+$ (Boehringer Mannheim, Indianapolis, IN).

RNA Extraction and Hybridization. Total RNA was prepared by guanidine isothiocyanate method as described (7). Aliquots of RNA (20 μg) were blotted onto nitrocellulose filters using a Minifold II slot blotter (Schleicher & Schuell, Keene, NH). The filters were then baked for 2 h at 80°C in a vacuum oven and hybridization of the ^{32}P-radiolabeled probe was done as described (7). A cDNA probe for *Eta-1* has been described previously (7). TCR-α probe was kindly provided by Dr. Anjana Rao (Dana Farber Cancer Institute) and IL-2 probe by Dr. Brigitte Huber (Tufts Medical School, Boston, MA).

Preparation of Ar5v Supernatants. Ar5v cells were maintained in DMEM containing 10% FCS, 2 mM L-glutamine, 50 μM 2-ME and 1% Con A–depleted spleen conditioned medium as a source of IL-2 (10). 1.5×10^7 cells were incubated with 20 ng/ml PMA in the same medium for 5 h, washed twice with DME and reincubated for an additional 10 h in DMEM without FCS containing PMA (20 ng/ml). Supernatants collected at the end of this incubation were centrifuged at 10,000 *g* for 20 min before further analysis.

Electrophoresis of Ar5v Supernatants and Extraction of P65 (eta-1) Protein from Polyacrylamide Gels. A modification (7) of the O'Farrell method (11) was used to resolve the supernatant proteins followed by elution of P65 protein from SDS-polyacrylamide gels. PMA-stimulated Ar5v supernatants were concentrated 30–40 fold by Centriprep-10 (Amicon Corp., Danvers, MA), loaded onto isoelectric focusing tube gels and focused for 2.5 h at 400 V. After electrophoresis in the first dimension, the 30-mm piece from the anodic end of the gels corresponding to pI 4–5 were cut and equilibrated for 20 min in the SDS-sample buffer. Gel slices in sets of three were loaded onto 10% SDS-polyacrylamide gels for second dimension electrophoresis. Prestained protein molecular weight markers were loaded before each gel slice. After electrophoresis, one set of gels was stained and P65 was located in the remaining gels using stained gel and prestained protein markers. Polyacrylamide gels containing P65 were cut into smaller pieces, ground, and extracted with 100 mM phosphate buffer, pH 7.0, at 37°C for 12 h. Supernatants were microfuged for 15 min to remove gel debris, concentrated, and either dialyzed extensively with phosphate buffer or passed through Extracti Gel-D detergent column (Pierce Chemical Co., Rockford, IL) to remove traces of SDS. An aliquot of the extracted protein was re-electrophoresed onto two-dimensional gels to confirm its purity. Protein concentration was estimated by the BCA protein assay reagent using BSA as standard (12). Material for control injections was prepared by isoelectric focusing of sample buffer without protein followed by electrophoresis under the same conditions as described above for the purification of P65 protein. Polyacrylamide pieces of these control gels were extracted from the pI 4.5, 65-kD mol mass region of the gel (similar to the location of P65 in the experiment gel) and processed as described for P65. P65 and control preparations were tested for the presence of endotoxin using Limulus amebocyte lysate (Associates of Cape Cod, Inc., Woods Hole, MA).

Sequence Analysis. After concentrated PMA-Ar5v supernatant fluid was electrophoresed under the conditions described above, proteins were electroblotted on nitrocellulose and stained with 0.1% Ponceau S (13). The p65 band was cut from nitrocellulose, subjected to trypsin digestion, and sequenced on ABI Protein Sequencer Model 477A as described (13).

Radioiodination of eta-1 (P65). Iodination of eta-1 (P65) was performed using the Iodobead method (14). Beads were washed twice with 50 mM sodium phosphate buffer, pH 7.4, and dried on Whatman No. 52 paper. 5 μl of ^{125}I solution (0.3 mCi/μl; New England Nuclear, Boston, MA) was added to dried beads; reaction was diluted to 200 μl with phosphate buffer and left at room temperature for 5 min. 60 μl of eta-1 (0.12 mg/ml) was added to the preloaded beads and incubated for 15 min at room temperature. The reaction was quenched

by adding 1 μl of freshly prepared 100 mM NaI and labeled protein was separated from the beads to terminate the reaction. ^{125}I-conjugated eta-1 and free iodine were then separated over disposable Excellulose desalting columns (Pierce Chemical Co., IL) equilibrated in PBS containing 0.25% gelatin (~300 bloom, Sigma Chemical Co., St. Louis, MO). Fractions in the void volume containing ^{125}I-eta-1 were collected and stored at 4°C. Greater than 90% of the radioactivity in these fractions was precipitable with 10% TCA and resolved as a single spot of 65 kD, pI 4.5, after repeat electrophoresis. The ^{125}I-eta-1 obtained by this procedure had a specific activity of 22.7 μCi/μg.

Binding Assays. Cells used for binding studies had 90% or greater viability. Binding for all cell types except RPM was measured as described (15). Briefly, different concentrations of ^{125}I-eta-1 in 250 μl of HBSS with 0.1% BSA, 2 mM L-glutamine (binding media) were added to cells and incubated for 1.5 h at 20°C with gentle rocking. Following reaction, cells were layered over 1 ml of a mixture of six parts of dioctylpthalate and four parts dibutylpthalate oil (Aldrich Chemical Co., Milwaukee, WI) and centrifuged for 30 min at 4°C. Radioactivity in the pellet and supernatant fractions was determined in a gamma counter (No. 5500B; Beckman Instruments, Fullerton, CA). Specific binding was defined as the fraction of total binding that was inhibited in the presence of a 200–400-fold molar excess of unlabeled eta-1. In the case of RPM, binding was determined in 96-well tissue culture plates as described (16). At the end of the 1.5-h incubation, unbound ligand was washed four times with 0.3 ml of binding medium. Cells were solubilized with 250 μl of 1% SDS and radioactivity of the cell lysate was quantitated as above. The 19-mer peptides were synthesized at Multiple Peptide Systems (San Diego, CA) and were 70–80% pure as judged by reverse-phase HPLC.

Histologic Analysis. Following the subcutaneous injection of eta-1 or control supernatant into a shaved right axillary region of BALB/c mice, epithelial tissue was removed at intervals of 3 and 24 h. After fixation in 10% formalin, tissue was embedded in paraffin, sectioned, and stained with hematoxylin and eosin or deparaffinized and stained with Giemsa in 10 mM sodium phosphate buffer, pH 7.4. To obtain relative quantitation of the number of cells present in the observed cellular inflammatory infiltrate, cells were enumerated in 20 random fields surrounding the injection site (histologically representing the dispersion of the dermal tissue at ×120). The number of leukocytes in each field was determined at ×400 using a calibrated micrometer grid. The different inflammatory cell types in the infiltrate were assessed using H+E- and Giemsa-stained tissue sections. 200–500 cells at the injection site were examined to determine the frequency of polymorphonuclear leukocytes (PMN), macrophages, mast cells, lymphocytes, and eosinophils in the cellular infiltrate.

Results

Definition and Purification of Natural eta-1 Protein. We determined the level of *Eta-1* RNA expression in a panel of murine T cell lines and clones. As expected from earlier studies, these cell lines did not express *Eta-1* under resting conditions as typified by murine T-cell tumor EL-4 (Fig. 1 *A*). An exception was a variant of an arsonate-responsive CD4$^+$ T-cell clone Ar5, termed Ar5v, which constitutively expressed high levels of *Eta-1* RNA before and after stimulation with PMA (Fig. 1, *A*, *B*). Analysis of the secreted products of resting and PMA-stimulated Ar5v cells was performed by two-dimensional gel electrophoresis under reducing conditions (Fig. 2). After ^{35}S-metabolic labeling, supernatant fluids of PMA-activated Ar5v cells contained a major band of 65 kD (referred to below as P65) having a pI of 4.5, the approximate size and charge of recombinant eta-1 (7). P65 was the predominant labeled protein in stimulated Ar5v supernatant fluids and accounted for ~10% of the total biosynthetically labeled material. Although resting and PMA-activated Ar5v cells had similar levels of *Eta-1* RNA, the supernatant fluids from the latter contained five- to eight-fold higher levels of P65 protein, possibly reflecting a requirement for PMA-dependent cellular changes required for optimal protein synthesis and/or secretion. Since the

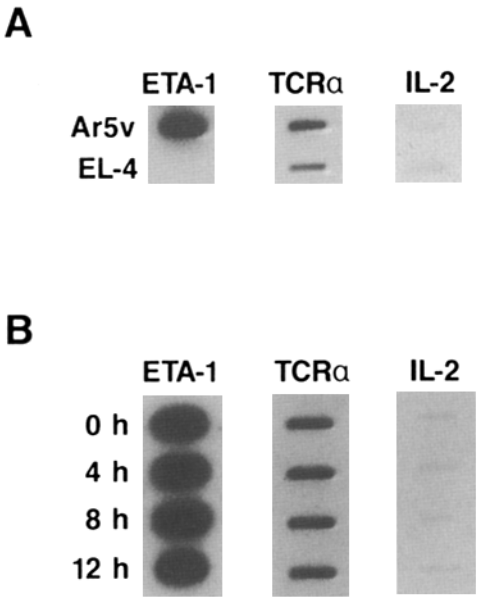

FIGURE 1. Expression of Eta-1 in Ar5v cells. A ^{32}P-labeled 1.7-kb Xho I fragment representing the *Eta-1* cDNA insert from pcD-Eta-1 vector was hybridized onto nitrocellulose filters containing 20 μg of total RNA (see Materials and Methods) from (*A*) resting Ar5v and EL-4 cells, and (*B*) Ar5v cells stimulated with PMA at 4, 8, and 12 h. Steady-state levels of TCR-α and IL-2 RNAs are shown as internal controls to denote constancy of total RNA blotted for each sample.

predicted eta-1 sequence lacked cysteine residues, we determined the migration of P65 under nonreducing conditions to rule out the possibility that P65 represented a reduced component of a larger S-S bonded molecule. No difference in the migration of P65 was noted after electrophoresis in two-dimensional gels under reducing or nonreducing conditions (not shown).

Further characterization of P65 was performed by microsequence analysis of the

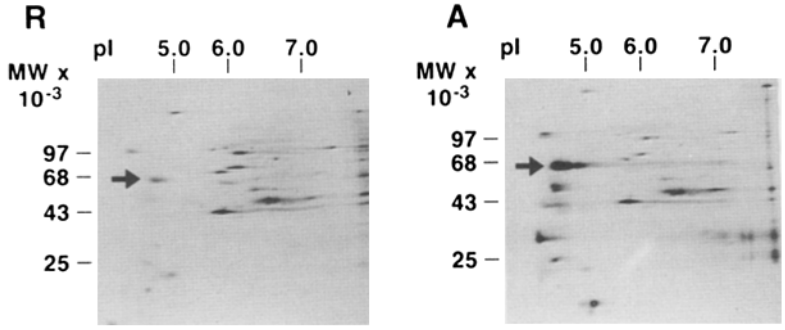

FIGURE 2. Analysis of proteins produced by Ar-5v T cells by two-dimensional gel electrophoresis. Biosynthetically labeled proteins in the supernatant fluids of resting Ar5v T-cells (*R*) and 10 h after activation with PMA (20 ng/ml) (*A*). Protein patterns are identical except for the increased expression of an acidic 65-kD (P65) protein together with 55- and 32-kD proteins after activation by PMA. P65 protein is denoted by an arrow. Cells were biosynthetically labeled by washing with methionine-free DMEM-L-Gln followed by incubation in the same medium supplemented with 100 μCi/ml of [^{35}S]methionine (800 Ci/mmol, New England Nuclear). 10 h later supernatants were collected and electrophoresed in two dimensions using a modification of the method of O'Farrell (11) (see Materials and Methods). The gels were treated with En^{3}hance (NEN), dried, and exposed for autoradiography. pI scale was determined using amyloglucosidase (pI 3.55), β-lactoglobulin A (pI 5.13), and myoglobin (pI 6.76, 7.16) as standard proteins.

isolated protein (see Materials and Methods). Since the NH_2 terminus of P65 was acetylated, the protein was subjected to trypsin digestion followed by elution of peptide fragments by HPLC. Microsequence analysis of a major peptide fragment in the HPLC elution profile indicated that this 19 amino acid long sequence was identical to a fragment of eta-1 representing positions 158–176 (Fig. 3, *A*, *B*) and did not match any other subsequences in the EMBL/GenBank or PIR Protein Sequence data base. These observations indicate that P65 represents naturally synthesized and secreted eta-1 protein. Estimation of the amount of eta-1 protein in supernatant fluid of Ar5v cells and in supernatant fluids of pcD-*Eta-1* transfected COS7m6 cells according to silver staining followed by densitometric scanning indicated that eta-1 expression was around 1–3 μg/ml in PMA-Ar5v supernatants. This concentration was 50–100-fold greater than the concentration of recombinant eta-1 in supernatants of transfected COS7m6 cells.

Comparison of Eta-1 Binding to Different Cell Types. The specificity of purified radiolabeled eta-1 binding to cells was measured in the presence or absence of a 400-fold molar excess of unlabeled purified eta-1. Substantial amounts of eta-1 bound to both RPM and to the macrophage-like cell line Wehi 3. In both cases, 70–80% of bound CPM was inhibited by unlabeled eta-1 (Fig. 4). Eta-1 did not specifically bind to the fibroblast cell line NIH3T3, nor to resting or activated Th cell clone O3 or Ar5.

Binding of eta-1 to Macrophages. To determine the affinity of eta-1 binding and number of eta-1 receptors on macrophages, RPM, and Wehi 3 cells were incubated with 80–1,000 pM ^{125}I-eta-1 for 90 min at 20°C. RPM or Wehi 3 cell-associated radioactivity increased until the concentration of ^{125}I-eta-1 was 500 pM, indicating saturable binding, and ~80% of this binding was inhibited by unlabeled eta-1 (Fig. 5). Scatchard analysis of these data indicated ~10,500 eta-1 receptors/RPM, each binding with an apparent K_d of 5.14×10^{-10} M. Analysis of the Wehi 3 cell line indicated that these cells had ~6,000 eta-1 receptors/cell and that these receptors had a dissociation constant similar to eta-1 receptors on RPM (K_d, 6.54×10^{-10} M).

Eta-1 Binds to Macrophages through an RGD Motif. The predicted amino acid sequence of eta-1 includes an RGD-tripeptide subsequence at positions 144–146 (7). This motif forms a critical part of the cellular binding site on a family of extracellular proteins that are involved in migration and cell–cell interaction (17). We synthesized a 19 amino acid long peptide corresponding to positions 136–154 of eta-1 that contained the RGD tripeptide subsequence (Fig. 3 *B*). A control peptide con-

A

Cyc	Residue		Pmole
1	SER	S	14.3
2	PHE	F	3.8
3	GLN	Q	3.1
4	VAL	V	2
5	SER	S	2.8
6	ASP	D	1.2
7	GLU	E	1.9
8	GLN	Q	1.5
9	TYR	Y	1.9
10	PRO	P	1.5
11	ASP	D	0.6
12	ALA	A	1
13	THR	T	0.7
14	ASP	D	1
15	GLU	E	0.7
16	[ASP	D]	0.1
17	[LEU	L]	1
18	(THR	T)	0
19	[SER	S]	0

B

127 QADTFTPIVPTVDVPNG[RGD]SLAYGLRSKSRSFQ
161 VSDEQYPDATDEDLTSHMKSGESKESLDVIPVA

FIGURE 3. Comparison of the amino acid sequence of a P65 peptide with the amino-acid sequence predicted from Eta-1 cDNA. (*A*) Confidence code of sequenced amino acids from a peptide of P65 tryptic digest is shown as high unless otherwise indicated: [ ] probable/reasonable, ( ) possible/low; (*B*) amino acid sequence predicted from *Eta-1* cDNA is shown; subsequence matching derived sequence of P65 peptide (positions 158–176) is underlined. The RGD subsequence representing the binding domain of eta-1 is boxed.

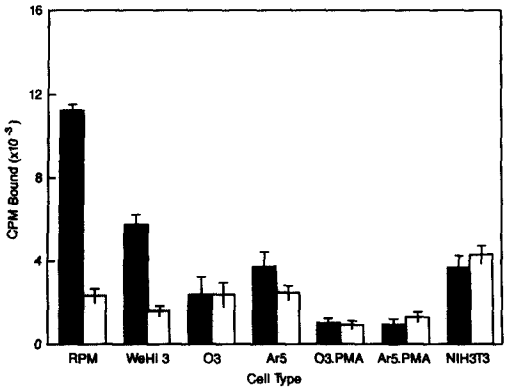

FIGURE 4. Binding of ^{125}I-eta-1 to different cell types. 2×10^5 cells were incubated in 0.25 ml of binding medium with 1×10^{-9} M of ^{125}I-eta-1 at 20°C for 1.5 h. Results shown represent cpm bound at saturation conditions. Nonspecific binding represents cpm bound in the presence of a 200–400-fold molar excess of unlabeled eta-1. (■) Total binding; (□) nonspecific binding.

tained two mutations where arginine (R, position 144) and aspartate (D, position 146) were both replaced with leucine (L). Incubation of RPM and Wehi 3 cells with an excess of the RGD-containing peptide but not the mutated peptide inhibited 85% of binding of ^{125}I-eta-1 to RPM and Wehi 3 (Fig. 6).

Effect of In Vivo Administration of eta-1: Histologic Analysis. These results, along with previous studies suggesting that eta-1 might affect macrophage responses in vivo (7), led us to determine whether in vivo inoculation of eta-1 might result in local accumulation of this cell type. Following intradermal injection of ~300 ng of eta-1 into BALB/c mice, a cellular infiltrate was observed at the injection site (as compared with the injection site of a control preparation) at 3 and 24 h (Table I, Fig.

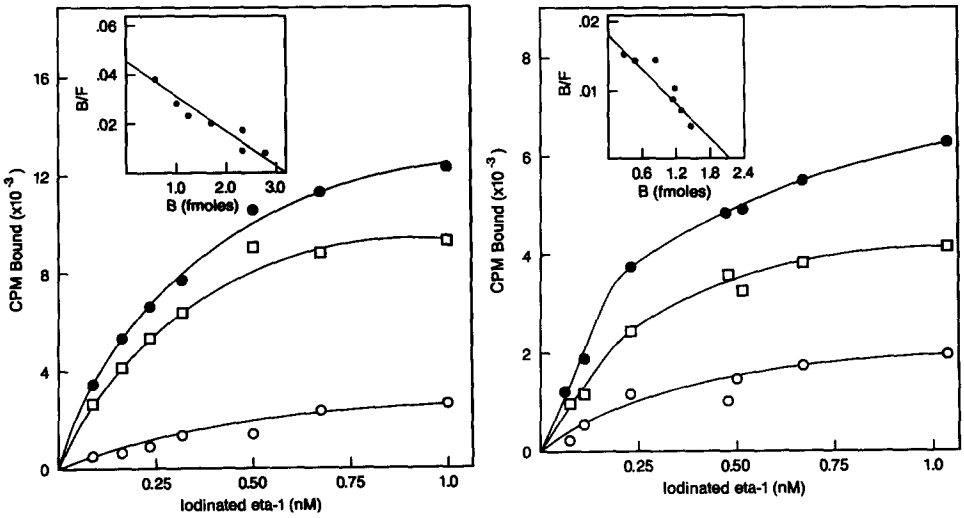

FIGURE 5. Saturation binding curves of radioiodinated eta-1 with (*A*) RPM and (*B*) Wehi 3 cells. Cells (2×10^5) were incubated with increasing concentrations of ^{125}I-eta-1 in 250 μl assay medium for 1.5 h at 20°C. Nonspecific binding (○) was determined in the presence of a 300–400-fold excess of unlabeled eta-1. Specific binding (□) represents the difference between total bound (●) and nonspecific binding. Inset, Scatchard plot of the same data. (*B*) bound eta-1, (*F*) free eta-1.

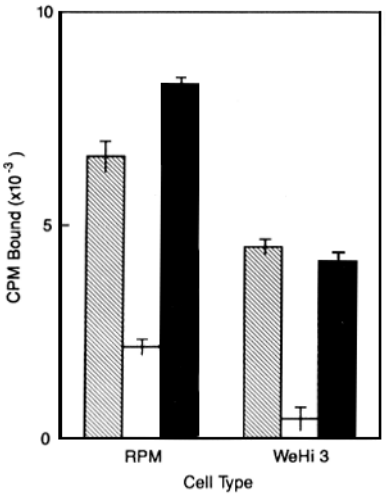

FIGURE 6. An RGD peptide inhibits eta-1 binding to macrophages. 2×10^5 cells were preincubated with 0.48 μM of RGD 19-mer or LGL 19-mer peptides along with 0.4 nM of ^{125}I-eta-1 for 1.5 h at 20°C. Specific binding of eta-1 is shown. (▨) ^{125}I-eta-1; (□) ^{125}I-eta-1 + RGD_{19mer}; (■) ^{125}I-eta-1 + LGL_{19mer}.

7, *A*, *B*). The numbers of inflammatory cells were elevated as early as 3 h after injection of eta-1 and at 24 h showed a 10-fold increase compared with control injection sites (Table I). The majority of cells in the cellular infiltrate were macrophages (Fig. 7, *C*, *D*) and the number of these cells 24 h after eta-1 injection was ~25-fold greater than in control injection sites. A significant, albeit less pronounced, elevation was also noted in the numbers of PMN, which increased fivefold after eta-1 injection. It was unlikely that the histologic reactions associated with eta-1 inoculation reflected the effects of contaminant endotoxin since the preparation of eta-1 used in these studies

TABLE I

Histologic Analysis of the Cellular Infiltrate following Subcutaneous Injection of Eta-1

	Number of cells/mm²*		Percent of cells/mm²‡				
	Mean	SE	PMN	MAC	LYM	MAST	EOS
Control (3 h)	19.4	1.1	61.1	29.1	0.6	8.3	0.6
eta-1 (3 h)	69.8	5.2	42.4	44.1	1.2	11.6	0.8
Control (24 h)	10.1	1.8	71.0	15.8	3.7	7.4	1.8
eta-1 (24 h)	99.7	5.2	35.2	56.8	1.9	5.0	0.5
			Number of cells/mm²				
		Total	PMN	MAC	LYM	MAST	EOS
Control (24 h)		10	7	2	0	1	0
eta-1 (24 h)		100	35	56	2	5	1

* Mean number of cells/mm² obtained from 20 random fields surrounding the injection site after staining with hematoxylin and eosin. Each group represents the results from three individual mice and the data shown represent one of three similar experiments.

‡ Differential analysis of the cells present in the inflammatory infiltrate after tissue sections were stained with hematoxylin + eosin or Giemsa to delineate nuclear configuration. The absolute numbers of each cell type/mm² are shown below.

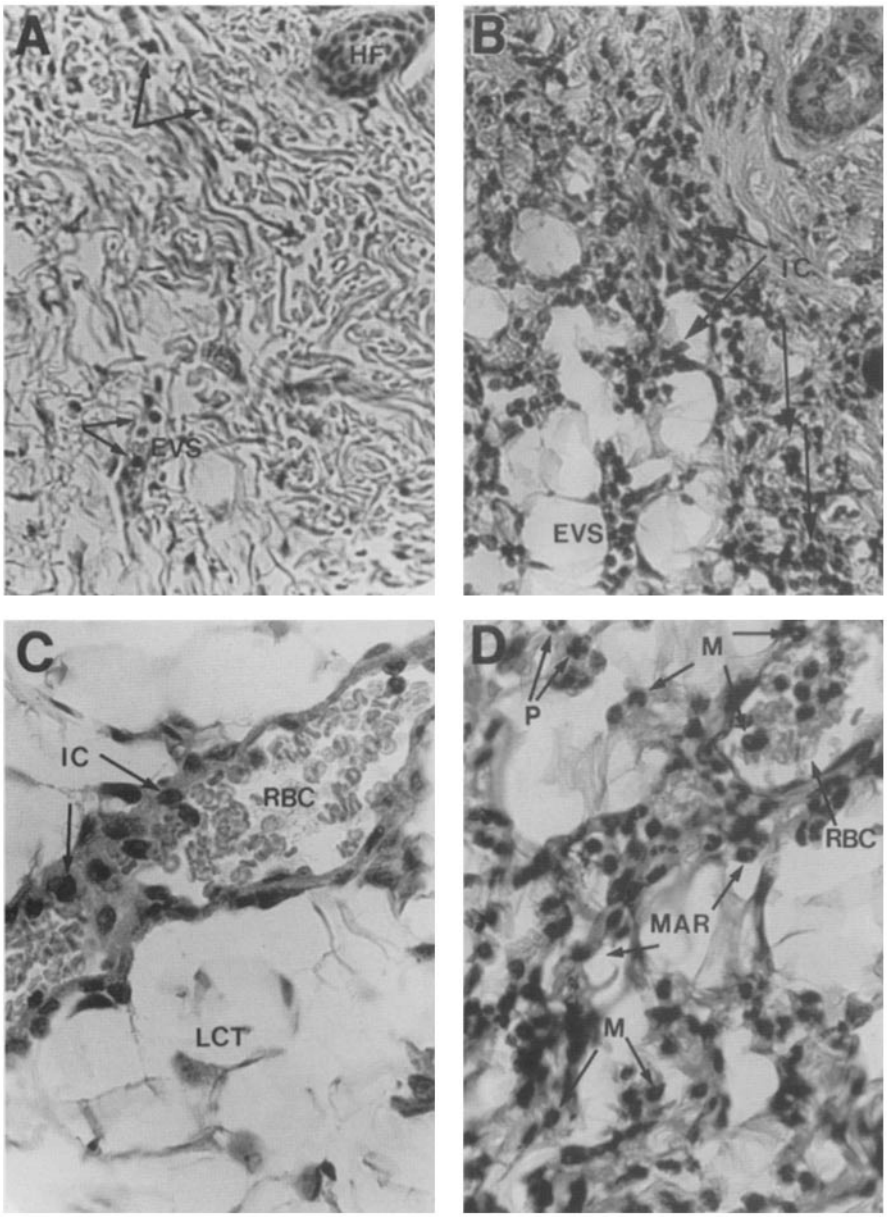

FIGURE 7. Histopathological analysis of cellular response following subcutaneous administration of eta-1. (*A*) 24 h following the subcutaneous injection of control supernatant, there was no recognizable inflammatory infiltrate into the local dermal tissue. Individual leukocytes were noted either in small endothelial lined vascular spaces or in the dermal loose connective tissue (×200). Arrows show leukocytes. (*HF*) hair follicle; (*EVS*) endothelial lined vascular spaces. (*B*) 24 h following the subcutaneous injection of 300 ng of eta-1, there was a dense inflammatory infiltrate of mononuclear cells and polymorphonuclear leukocytes within the interstices of loose connective tissue and adipocytes (×200). Arrows show inflammatory cells (*IC*). (*C*) Higher magnification (×400) of an endothelial-lined vascular space located at the subcutaneous injection site of control material shows a paucity of inflammatory cells in the loose connective tissue surrounding a blood vessel. (*RBC*) red blood cells; (*IC*) inflammatory cells; (*LCT*) loose connec-

contained <0.1 ng/g endotoxin, the lower limit of detection using a Limulus lysate assay.

Discussion

We have previously described a murine cDNA, termed *Eta-1*, that is strongly expressed after activation of T cells (7). The *Eta-1* gene maps to the *Ric* locus on chromosome 5, which confers resistance to infection by an obligate intracellular bacterium and encodes a secreted protein that includes an RGD-containing cell-binding domain of the extracellular matrix protein similar to that of fibronectin (7, 17).

Previous studies of *Eta-1* have suggested that although it is secreted by T cells, it confers resistance to bacterial infection in vivo by affecting the ability of macrophages to migrate to the site of infection and/or to ingest and destroy bacteria (7). We therefore attempted to determine whether macrophages expressed eta-1 receptors. Although recombinant *Eta-1* has been expressed in COS cells (7), the levels of recombinant eta-1 expression in transfectant cell supernatant fluids (picograms to nanograms/milliliter) hindered attempts at purification of the eta-1 protein for binding and functional studies. We screened a variety of T cell lines to identify a cell producing high levels of natural eta-1. This resulted in purification of eta-1 from supernatant fluids of a murine $CD4^+$ Th cell clone in sufficient amounts for studies of its interaction with macrophages in vitro and its ability to induce a cellular inflammatory response in vivo.

We used the radioiodinated eta-1 protein purified by extraction from polyacrylamide gels for cell binding studies. Elution of proteins from acrylamide gels proved to be a rapid method for obtaining a homogenous preparation of eta-1 that retained binding activity in vitro and macrophage recruitment activity in vivo. This approach has been used previously for preparing biologically active IFN-γ (18), IL-1 (19), and MDGF (20).

Eta-1 bound to peritoneal macrophages explanted from mice and to the macrophage-like cell line Wehi 3, but not to the fibroblast cell line NIH3T3 nor to two Th clones. Binding to macrophages was specific, saturable, and displayed high affinity kinetics. The observed K_d of eta-1 was 5×10^{-10} M for RPM and Wehi 3 cells and the number of eta-1 receptors was $\sim 10^4$/cell, about the same as that detected for IFN-γ (21). The linear Scatchard plot that describes binding kinetics indicates a noncooperative interaction between eta-1 and its receptor on macrophages, suggesting the presence of a single class of eta-1 receptor for this cell type. By contrast, macrophages appear to express both low and high affinity forms of IFN-γ receptor (22). The affinity of the eta-1 receptor described in this report is similar to the high affinity form of the IFN-γ receptor and about one log higher than the low affinity gamma-IFN receptor on macrophages (21, 22). We have not determined whether other leukocytes that participate in inflammatory responses such as mast cells and PMN also express eta-1 receptors. The increase in the frequency of the latter cell type in cellular infiltrates following eta-1 administration in vivo suggests that this cell type may also bear eta-1

tive tissue. (*D*) The injection site of eta-1 shows substantial margination of inflammatory cells exiting from the vascular lined spaces. These cells are also noted within the vessel (×400). (*M*) mononuclear cells; (*P*) polymorphonuclear leukocytes; (*MAR*) margination; (*RBC*) red blood cells.

receptors or that the interaction between eta-1 and macrophages may result in the elaboration of material that results in an accumulation of PMN.

Eta-1 includes an Arg-Gly-Asp (RGD) subsequence that marks a family of extracellular matrix molecules that induce changes in cellular motility and/or differentiation (7, 17). In most cases, this subsequence forms a critical part of the protein's binding site to cell surface receptors. Although the positive and negative charges of the Arg and Asp residues are necessary for binding, additional structural properties of flanking sequences are required to ensure cell binding (23, 24). We therefore synthesized a 19-mer peptide that represented the RGD domain of eta-1. This peptide, but not a peptide with a mutated RGD site (Arg-Gly-Asp to Leu-Gly-Leu), inhibited eta-1 binding to macrophages, suggesting that the RGD domain of eta-1 is essential to its binding to macrophages. Moreover, preliminary studies indicate that the RGD peptide, but not a mutated peptide, inhibit the induction of a cellular infiltrate by eta-1 in vivo, supporting the view that this portion of eta-1 represents a critical site allowing physiological interaction of eta-1 with inflammatory cells. We do not know whether the in vivo effects of eta-1 reflect increased macrophage migration out of the vascular space into surrounding tissue or in situ proliferation of tissue macrophages. We favor the former possibility because of the prominence of extravasating macrophages located at the margins of small vessels on histologic examination. According to this hypothesis, macrophages making contact with eta-1 at the spaces between endothelial cells and epimyoepithelial cells migrate out of blood vessels into extravascular tissue across an eta-1 gradient. Further studies of potential chemotactic activity of eta-1 in vitro and the role of the RGD-containing subsequence of the molecule are required to test this idea.

Macrophages express several biologically active receptors, including Mac-1, LFA-1, and p150,95, which are structurally similar to the fibronectin receptor, and may in principle, bind to extracellular molecules that express RGD-binding domains (25). Attachment of fibronectin to macrophages via an RGD-site can result in an increase in receptors for the Fc portion of Ig and for C3b proteins (26, 27). Studies are in progress to determine the in vitro effects of eta-1 on macrophage physiology and to define the surface structure(s) that function as the macrophage eta-1 receptor.

Summary

The *Eta-1* gene specifies a secreted product of activated T cells and is associated with genetic resistance to infection by an obligate intracellular bacterium. Previous studies have suggested that eta-1 might affect the ability of macrophages to migrate to the site of bacterial infection and/or to inhibit intracellular bacterial growth. We therefore examined the interaction of eta-1 with macrophages in vitro and in vivo. We find that macrophages express $\sim 10^4$ eta-1 receptors/cell and each receptor has a K_d of $\sim 5 \times 10^{-10}$ M. The subsequence of eta-1 containing an RGD motif is required for binding because a synthetic peptide containing the eta-1 RGD domain inhibited protein attachment to macrophages. We also found that subcutaneous inoculation of mice with eta-1 resulted in a cellular infiltrate comprised primarily of macrophages. We propose that the interaction between eta-1 and its receptor on macrophages results in a change in macrophage physiology resulting in accumulation of these cells at extravascular sites.

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